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TITLE: Itraconazol, an Antifungal and a Hedgehog Pathway Inhibitor for Treatment of Prostate Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
A commonly used antifungal, Itraconazol targets a pathway that is also upregulated in human prostate cancer. It was observed that Itraconazole synergizes with cyclopamine to induce superior therapeutic effects. Cyclopamine is toxic, however, when combined with itraconazole the dose requirement for each drug was considerably reduced. This combination therefore has the potential to be more effective and at the same time less toxic. The most important aspect of this finding is that these are existing drugs whose safety and toxicological profiles are known. This project seeks to investigate the efficacy of Itraconazol against prostate cancer with an intention to accelerate its rapid translation into human clinical trials. We are in the process of identifying a combination of drugs for the treatment of advanced prostate cancer. Although these are preclinical studies, however, if the efficacy of the drug can be established against prostate cancer in this proof-of-principle study then clinical trials could be immediately started based on the fact that these are existing drugs with all the information about toxicity and dose. The drugs do not have to undergo the mandatory initial trials to establish dose and toxicity. This report outlines our progress to date.

15. SUBJECT TERMS

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The text of the report must include all sections addressed in the table of contents to include the following. **DO** include the bolded section headings, but **DO NOT** include the *italicized* descriptions of section contents in your submitted reports.

1. **INTRODUCTION:**

   Hedgehog (Hh) signaling is activated in advanced prostate cancer (PCa) and is required for proliferation, viability, and invasive behavior. The levels of Hh activity also correlate with the severity of the tumor and are both necessary and sufficient for metastatic behavior. Blockade of Hh signaling leads to tumor shrinkage and remission in preclinical tumor models. We hypothesize that targeting Hh pathway activation in advanced PCa will result in decreased Hh signaling and subsequent inhibition of prostate tumorigenesis. Current Hh inhibitors such as cyclopamine present with severe side effects in non-tumor tissues. Itraconazole, a commonly used antifungal agent with a well-known safety profile was observed to inhibit the hedgehog (Hh) pathway by acting on the essential Hh component Smoothened (SMO) through a mechanism distinct from cyclopamine and other SMO antagonists. We will evaluate the effect of itraconazole alone and in combination with cyclopamine, on the growth and metastasis of human PCa *in vitro* and *in vivo* with an intention to accelerate their rapid translation into human clinical trials. We propose to study the effect of itraconazole and cyclopamine alone and in combination against PCa both under *in vitro* and *in vivo* conditions. We will evaluate the effect of the combination on the growth and metastasis of human PCa cells representative of the advanced disease; the effect of the combination, on the growth and metastasis of human PCa cells implanted orthotopically and subcutaneously in male athymic nude mice. PC-3 cells labeled with luciferase and green fluorescent protein will be used to monitor the effect of drugs and the metastatic dissemination pattern to various tissues. We will investigate the effect of the combination on the progression of PCa in the PTEN knockout mouse model that recapitulates features of advanced human PCa. The data obtained will be extremely valuable in providing information on the usefulness of novel Hh signaling inhibitors against PCa for their rapid translation into human clinical trials.

2. **KEYWORDS:**

   Prostate cancer, itraconazole, cyclopamine, combination, hedgehog, signaling, antifungal, PTEN, metastasis, cell growth, viability

3. **ACCOMPLISHMENTS:**
   o **What were the major goals of the project?**

   To evaluate the effect of itraconazole on the growth and metastasis of human prostate cancer cells in vitro. **Months 1-3**

   To explore the sensitivity of prostate cancer cells to cell growth inhibition by itraconazole treatment **Months 4-8**

   To examine the mode of cell death by itraconazole treatment **Months 5-10 and repeat experiments with other prostate cancer cell lines**

   To determine the effect of itraconazole on metastatic potential of human prostate cancer cells **Months 6-12 and repeat experiments with other prostate cancer cell lines**

   To establish the role of Hh signaling as a target of itraconazole and its functional relevance in prostate cancer cells

- o **What was accomplished under these goals?**
  - ▪ 1) Major activities: Human PCa cell lines representative of the androgen-sensitive and insensitive status such as DU145, PC-3, 22Rv1 and C4-2B were treated with itraconazole and cyclopamine for 24 and 48 hours. Normal prostate epithelial cells (RWPE) were also treated in a similar manner to ascertain effect on normal cells. Cell growth and viability were ascertained by MTT assay. The purpose of these studies would help to identify sensitivity of PCa cells to itraconazole and cyclopamine and identify the best dose combination for all subsequent studies and further categorize cells as sensitive and/or resistant to treatment with Hh inhibitors. Normal human prostate epithelial cells were also treated with itraconazole and cyclopamine to ascertain if non-cancerous cells are affected by Hh inhibition.
  - ▪ 2) Specific objectives: Explore the sensitivity of PCa cells to cell growth inhibition by itraconazole and cyclopamine.
  - ▪ 3) Significant results: Existing studies including our own observations suggest that Hh signaling is active during the development and progression of prostate cancer in humans. Specific Hh inhibitors inhibit the growth of prostate cancer cells lines including PC3, DU145 and 22Rv1 cells. However, clinical application of Hh inhibitors has been slow due to the fact that available inhibitors are associated with severe side effects. The discovery of itraconazole provides an opportunity to target the Hh signaling in prostate cancer and help its rapid translation into the clinic. Itraconazole synergizes with the known Hh inhibitor cyclopamine and the combination results in several fold lower dose requirements.

    We undertook studies to ascertain the effect of itraconazole and cyclopamine on growth kinetics using several prostate cancer cells including a normal prostate epithelial cell line RWPE. The primary purpose was to see the effect on growth and then to understand the dose that would be ideal for treatment when the two drugs are combined.

    Cell Growth Analysis: Cells were seeded (6 x 10^4 cells/2 mL) in 12-well plates (24 hours), and treated with itraconazole (0-10 μM) for 24 and 48 hours or cyclopamine (0-50 nM) for 24 and 48 hours. Cell growth was determined by MTT assay according to the manufacturer's protocol. Briefly, after treatment, cells were then treated with media containing MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 2 hours. Metabolically intact cells cleave tetrazolium salts by the succinate-tetrazolium reductase system to purple Formazan crystals and lesser the formation of crystals greater is the inhibition of cell growth. Afterward, the MTT solution was removed, and the blue crystalline precipitate internalized by the cells was dissolved in DMSO. Finally, plates were placed in a plate reader to measure absorbance at 570 nm. Both itraconazole and cyclopamine significantly affected cell growth.

    To investigate the mode of cell death whether apoptosis or necrosis, we examined the activation of poly ADP-ribose polymerase (PARP), a key effector molecule of apoptosis. We observed that itraconazole largely resulted in apoptotic cell death whereas cell death by cyclopamine was mostly necrotic. Itraconazole alone increased cleavage of PARP. We studied other molecules involved during the initiation and execution of apoptosis. Levels of pro-apoptotic Bax were increased by itraconazole and at the same time, levels of anti-apoptotic Mcl-1 decreased. Overexpression of proliferation markers such as proliferating cell nuclear antigen (PCNA) and Ki67 are considered prognostic biomarkers for various types of cancers. The levels of PCNA expression was inhibited in the cells treated with either itraconazole or cyclopamine. These data suggest that both drugs affect cell proliferation.
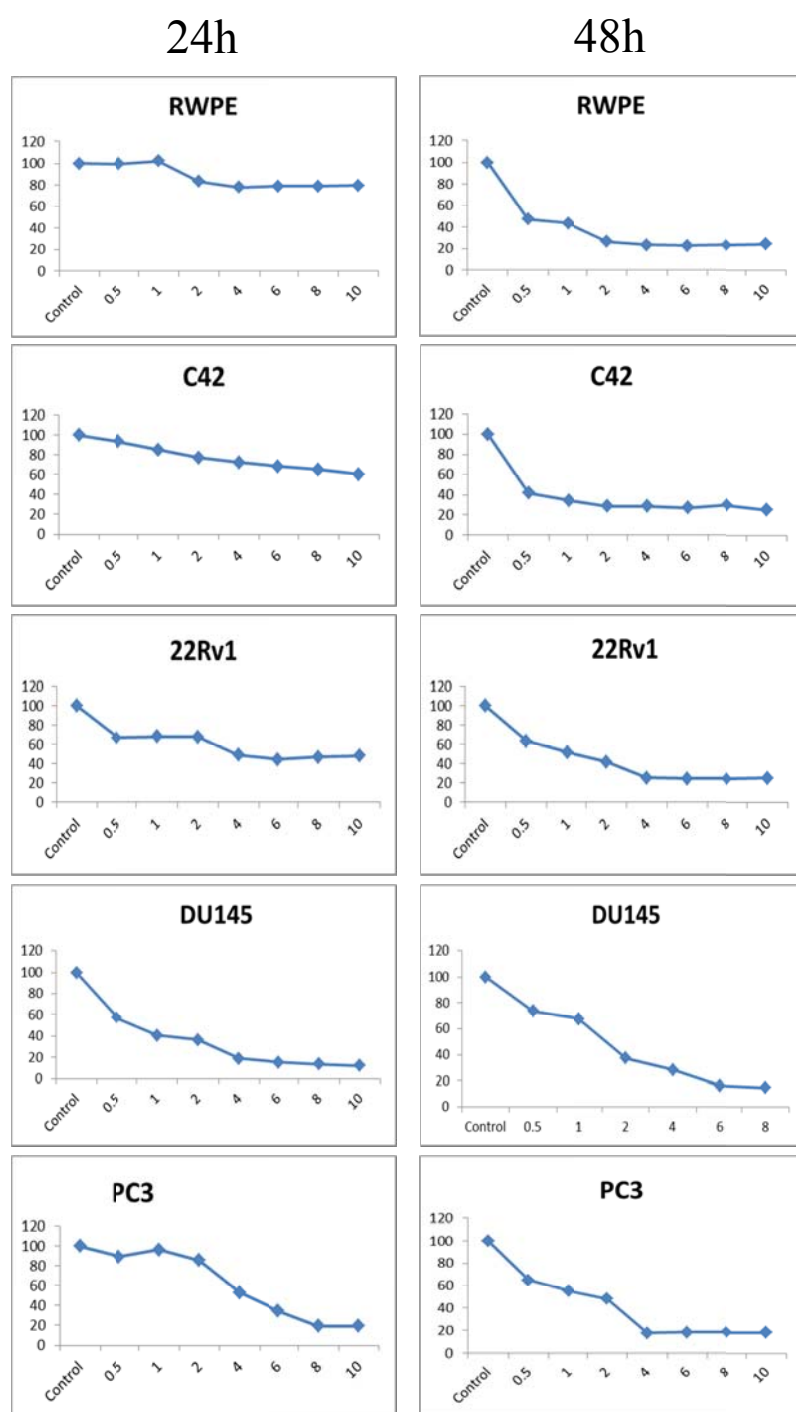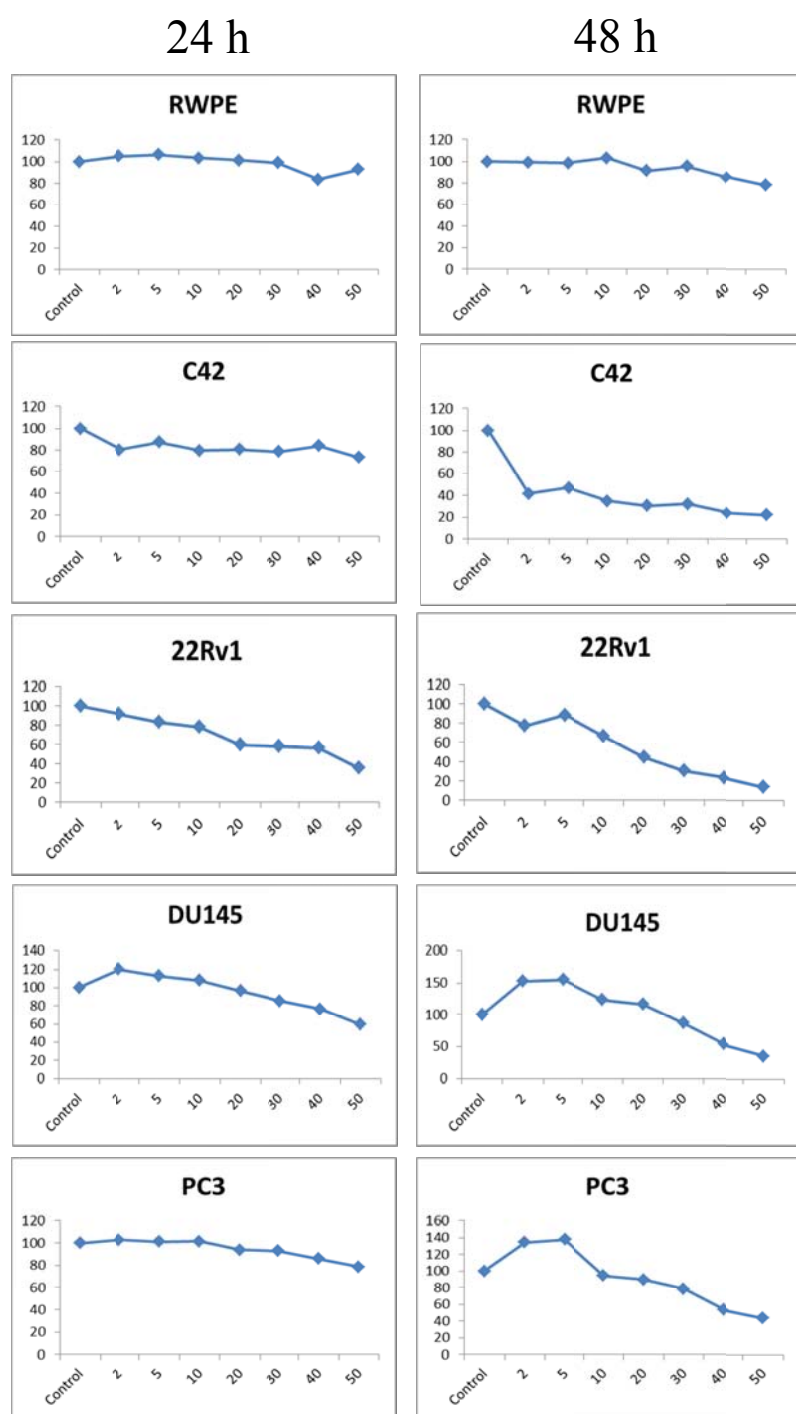
Figure 1: Effect of itraconazole on prostate cancer cell growth. Prostate cancer cells were treated with indicated concentrations of itraconazole for 24 and 48 hours. After the completion of the time, cells were processed for cell growth analysis using the MTT assay. Cells were incubated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 2 hours. Metabolically intact cells cleave tetrazolium salts by the succinate-tetrazolium reductase system to purple Formazan crystals. The lesser the formation of crystals greater is the inhibition of cell growth.

24 h   **48 h**

Cell Growth (%Control)

Figure 2: Effect of cyclopamine on prostate cancer cell growth. Prostate cancer cells were treated with indicated concentrations of cyclopamine for 24 and 48 hours. After the completion of the time, cells were processed for cell growth analysis using the MTT assay. Cells were incubated with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 2 hours. Metabolically intact cells cleave tetrazolium salts by the succinate-tetrazolium reductase system to purple Formazan crystals. The lesser the formation of crystals greater is the inhibition of cell growth.

- *4) Other achievements.* The primary purpose of our initial experiments was to study the effect of each individual drug on growth and cell viability with the objective to identify ideal doses for use in combination. An unexpected observation was the unusual growth inhibitory effect of itraconazole on normal prostate cells only at 48 hours. We are repeating these experiments to understand whether these are real observations or affected by other factors. Activation of matrix metalloproteinase (MMPs), urokinase plasminogen activator (uPA), and angiogenic factors (VEGF and CD31) are shown to promote prostate cancer metastasis into distant organs. A possibility was explored whether itraconazole and cyclopamine alone inhibit the expression of MMP2, MMP9 and CD31 in cells. Western blot analysis indicated a significant decrease in MMP2, MMP9 expression with each drug. Similarly, these drugs also resulted in decreased expression of CD31 and other metastatic markers such as uPA.

- **What opportunities for training and professional development has the project provided?**
- "Nothing to Report."
- **How were the results disseminated to communities of interest?**
- "Nothing to Report."
- **What do you plan to do during the next reporting period to accomplish the goals?**
- Repeat experiments with other prostate cancer cell lines
- Investigate the effect of targeting Hh signaling on markers that determine epithelial to mesenchymal transition (EMT).
- Review all in vitro experiments and repeat any experiments in necessary
- Investigate the effect of itraconazole on the growth and metastasis of human prostate cancer cells implanted in athymic nude mice.
- Prepare C4-2B cell line stably transfected with the luciferase (luc) expression vector pCMVluc. Prepare nude mice xenograft for following tumor studies:
- Tumor growth studies
- Metastasis monitoring
- In vivo cell proliferation studies
- Metastatic studies
- Immunohistochemistry
- Review nude mice data and repeat experiments if necessary

4. **IMPACT:**
- **What was the impact on the development of the principal discipline(s) of the project?**
- "Nothing to Report."
- **What was the impact on other disciplines?**
- "Nothing to Report."
- **What was the impact on technology transfer?**
- "Nothing to Report."
- **What was the impact on society beyond science and technology?**
- \"Nothing to Report."

5. **CHANGES/PROBLEMS:**
  - **Changes in approach and reasons for change**
    - "Nothing to Report"
  - **Actual or anticipated problems or delays and actions or plans to resolve them**
    - "Nothing to Report"
  - **Changes that had a significant impact on expenditures**
    - "Nothing to Report"
  - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - "Nothing to Report"
  - **Significant changes in use or care of human subjects**
    - "Nothing to Report"
  - **Significant changes in use or care of vertebrate animals.**

- ▪ *"Nothing to Report"*
    - o **Significant changes in use of biohazards and/or select agents**
6. **PRODUCTS:** *"Nothing to Report."*
7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS—**

Name:	Project Role:	Researcher Identifier	Nearest person month worked:	Contribution to Project:	Funding Support:
Hasan Mukhtar	PI	None	18	Overall project administration	This grant
Vaqar Adhami	Co-Investigator	3.0	0.36	Contributor, PCa cell sensitivity, growth kinetics	This grant
Mohammed Imran Khan	Postdoctoral Researcher	None	9.0	Contributor,PCa cell growth kinetics	This grant

- o **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - ▪ "Nothing to Report."
- o **What other organizations were involved as partners?**
    - ▪ "Nothing to Report"
8. **SPECIAL REPORTING REQUIREMENTS**—Nothing to report
9. **APPENDICES:** None.